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Microbial production of l-aldonolactone from l-aldohexose

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Microbial Production of L-Aldonolactone from L-Aldohexose

The present invention is directed to a process for producing a L-aldonolactone from a L-aldohexose by a microorganism belonging to the genus *Pseudomonas* or the genus *Gluconobacter*.

The L-aldonolactones L-gulono-1,4-lactone and L-galactono-1,4-lactone, respectively, are intermediates in the biosynthesis of L-ascorbic acid (AsA) by animals and plants. The proposed pathway for the synthesis of L-ascorbic acid in animals starts from D-glucose and goes on via the intermediates D-glucose-6-phospate, D-glucose-1-phosphate, UDP-D-glucose, UDP-D-glucuronic acid, D-glucuronic acid, L-gulonic acid, L-gulono-1,4-lactone and 2-keto-L-gulono-1,4-lactone to the endproduct L-ascorbic acid. The proposed pathway for the synthesis of L-ascorbic acid in plants starts from D-glucose and goes on via the intermediates D-glucose-6-phospate, D-fructose-6-phospate, D-mannose-6-phosphate, GDP-D-mannose, GDP-L-galactose, L-galactose-1-phosphate, L-galactose, L-galactono-1,4-lactone and 2-keto-L-galactono-1,4-lactone to the endproduct L-ascorbic acid.

Feasibility studies on the biotechnological synthesis of AsA were performed for many years since the "Reichstein method" was established in 1934. The microorganisms *Gluconobacter oxydans* DSM 4025, *Candida albicans* and *Saccharomyces cerevisiae* oxidize L-galactono-1,4-lactone to AsA. *Saccharomyces cerevisiae* and *Candida albicans* possess a D-arabinose dehydrogenase catalyzing the production of D-arabinono-1,4-lactone and L-galactono-1,4-lactone from D-arabinose and L-galactose, respectively. However, there were no reports describing the possibility of biological AsA production from another L-hexose as intermediate, that is, L-idose, L-gulose, and L-talose, with a configuration corresponding to that of AsA (C4 and C5 positions).

The present invention provides a process for the production of L-aldonolactone from L-aldohexose, especially for producing L-gulono-1,4-lactone or L-gulonic acid from

L-gulose and producing L-galactono-1,4-lactone or L-galactonic acid from L-galactose by a microorganism capable of producing L-aldonolactone from L-aldohexose.

L-Aldohexoses like L-gulose, L-galactose, L-idose, and L-talose are rare sugars which are basically produced by chemical methods and are commercially high-cost compounds. However, biological preparations for L-gulose and L-galactose have been recently reported. L-Gulose production from D-sorbitol by Enzyme A of *G. oxydans* DSM 4025 was reported in EP 832,974. L-Gulose production from L-sorbose by L-ribose isomerase was disclosed in US 6,037,153 L-galactose production from L-sorbose is reported by Izumori et al. (2001 Annual Meeting of the Society for Bioscience and Bioengineering, Japan). In this process, they combined two enzymatic processes consisting of "L-sorbose to L-tagatose" reaction with L-tagatose epimerase of Pseudomonas cichorii ST-24 (US 5,811,271) and "L-tagatose to L-galactose" reaction with D-arabinose isomerase of *Bacillus stearothermophilus* strain 14a.

Microorganisms capable of producing L-aldonolactone from L-aldohexose may be selected from the genera *Pseudomonas* and *Gluconobacter*, e.g. *P. putida*, e.g., *P. putida* ATCC 21812, and *G. oxydans*, e.g., *G. oxydans* IFO 3293. The microorganism may also be a biologically and/or taxonomically homogeneous culture of a microorganism having the identifying characteristics of *Pseudomonas putida* ATCC 21812 or *Gluconobacter oxydans* IFO3293.

The present invention, therefore, provides a process for the production of L-aldonolactone from L-aldohexose, especially for producing L-gulono-1,4-lactone or L-gulonic acid from L-gulose, or L-galactono-1,4-lactone or L-galactonic acid from L-galactose by a microorganism belonging to the genera *Pseudomonas* or *Gluconobacter* capable of producing L-aldonolactone from L-aldohexose in a growing culture or a resting cell reaction, and isolating the L-aldonolactone from the reaction mixture.

In the present invention, mutants of the above mentioned strains can also be used. The mutant used in the present invention can be induced by treating a wild type strain with a mutagen such as ultraviolet-ray, X-ray, γ-ray, nitrous acid or other suitable mutagens, or can be obtained by isolating a clone occurring by spontaneous mutation thereof in any of the ways per se well known for the purpose by one skilled art.

The microorganisms may be cultured in an aqueous medium supplemented with appropriate nutrients under aerobic conditions. The cultivation may be conducted at a pH between about 1.0 and 9.0, preferably between about 2.0 and 8.0. While the cultivation

period varies depending on pH, temperature and nutrient medium used, usually 1 to 120 hours will bring about favorable results. A preferred temperature range for carrying out the cultivation is from about 13°C to 45°C preferably from about 18°C to 42°C.

As used herein, "L-gulono-1,4-lactone (and its acid form, L-gulonic acid)" or "L-galactono-1,4-lactone (and its acid form, L-galactonic acid)" means co-existing mixture of the lactone form together with the acid form as the result of physicochemical equilibrium.

The concentration of L- aldohexose in a reaction mixture can vary depending on other reaction conditions, but, in general, is between 1 g/l and 300 g/l, preferably between 10 g/l and 200g/l.

The reaction can be conducted aerobically.

It is usually required that the culture medium contains such nutrients as assimilable carbon sources, digestible nitrogen sources and inorganic substances, vitamins, trace elements and other growth promoting factors. Examples of assimilable carbon sources include glycerol, D-glucose, D-mannitol, D-fructose, D-arabitol, D-sorbitol and L-sorbose.

Various organic or inorganic substances may also be used as nitrogen sources, such as yeast extract, meat extract, peptone, casein, corn steep liquor, urea, amino acids, nitrates, ammonium salts and the like. As inorganic substances, magnesium sulfate, potassium phosphate, ferrous and ferric chlorides, calcium carbonate and the like may be used.

After the reaction, L-aldonolactone may be recovered from the reaction mixture by the combination of various kinds of chromatography, for example, thin layer chromatography, adsorption chromatography, ion-exchange chromatography, gel filtration chromatography or high performance liquid chromatography. The reaction product can also be used as a substrate for a further reaction as it is in the reaction mixture of this invention without purification.

The following examples are provided to further illustrate the process of the present invention. These examples are illustrative only and are not intended to limit the scope of the invention in any way.

Example 1: Production of L-gulono-1,4-lactone from L-gulose by *P. putida* or *G. oxydans*

P. putida ATCC21812 and *G. oxydans* IFO 3293 were grown on MB agar medium consisting of 2.5 % mannitol, 0.5 % yeast extract (Difco), and 0.3 % Bactopeptone (Difco) at 30°C for 48 hours. The resulting cells were used for a resting cell reaction. The reaction

mixture (1 ml) consisting of 2 % L-gulose, 0.3 % NaCl, 1 % $CaCO_3$ and 1 mM phenazine methosulfate was incubated at room temperature for 17 hours. The produced amounts of L-gulono-1,4-lactone and L-gulonic acid were assayed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) as summarized in Table 1. The TLC assay was done with silica gel (Kiesel gel $60F_{254}$, 0.25 mm, Merck), the solvent system consisting of n-propanol-H_2O-1% H_3PO_4-HCOOH (400:100:10:1). The HPLC assay was done at 210 nm with a YMC-Pack Polyamine II column (150 x 4.6 mm I.D.; YMC CO., Ltd., Kyoto, Japan) and with acetonitrile–50 mM $NH_4H_2PO_4$ (67:33). The TLC plate was sprayed with 0.5 % KIO_4 solution and then sprayed with the mixture of an equal volume of tetrabase-saturated 2N CH_3COOH and 15 % $MnSO_4$ solution. The substrate, L-gulono-1,4-lactone and L-gulonic acid were detected as white spots.

Table 1: Tube resting reaction with L-gulose as a substrate

Strain	TLC		HPLC (mM)
	L-GuL	L-GuA	L-GuL+LGuA
Pseudomonas putida ATCC 21812	nd	++	16.5
Gluconobacter oxydans IFO 3293	nd	+	5.2
No cells	nd	nd	nd

L-GuL: L-gulono-1,4-lactone; L-GuA: L-gulonic acid; ++: more than 5 mM; + 5 mM or less; nd: not detectable

The reactions with L-gulose as the substrate were also done in a mini-resting-cell reaction with 100 µl reaction mixture consisting of 2 % L-gulose, 0.3 % NaCl, 1 % $CaCO_3$. *Escherichia coli* HB101 grown on Luria Bertani (LB) agar at 37°C for 1 day was also used in this reaction. Amounts of produced L-gulono-1,4-lactone and L-gulonic acid are shown in Table 2.

Table 2: Mini-resting reaction with L-gulose as a substrate

Strain	TLC	
	L-GuL	L-GuA
Pseudomonas putida ATCC 21812	nd	+
Gluconobacter oxydans IFO 3293	+	+
Escherichia coli HB101	nd	nd
No cells	nd	nd

Example 2: Production of L-galactono-1,4-lactone from L-galactose

P. putida ATCC21812 and *G. oxydans* IFO 3293 were grown on MB agar plate at 30°C for 48 hours. *Saccharomyces cerevisiae* ATCC 9763 was grown on the YN medium (Difco) with 2 % D-glucose and 1.8 % agar at 30°C for 48 hours. *E. coli* HB101 grown on Luria Bertani (LB) agar at 37°C for 1 day was also used in this reaction. The resulting cells were used for a resting cell reaction. The reaction mixture (100 µl) consisted of 2 % L-galactose, 0.3 % NaCl, 1 % $CaCO_3$ and the cells (OD600=ca. 20) was incubated at room temperature for 23 hours. The produced amounts of L-galactono-1,4-lactone and L-galactonic acid were assayed by TLC and HPLC as summarized in Table 3. *P. putida* ATCC 9763 and *G. oxydans* IFO 3293 produced significantly more L-galactono-1,4-lactone together with L-galactonic acid than *S. cerevisiae* ATCC 9763 and *E. coli* HB101, both of which produced undetectable amounts of L-galactono-1,4-lactone and L-galactonic acid.

Table 3: Mini-resting reaction with L-galactose as a substrate

Strain	TLC		HPLC (mM)
	L-GaL	L-GaA	L-GaL + LGaA
Pseudomonas putida ATCC 21812	++	++	75.8
Gluconobacter oxydans IFO 3293	+	+	10.0
Saccharomyces cerevisiae ATCC 9763	nd	nd	nd
Escherichia coli HB101	nd	nd	nd
No cells	nd	nd	nd

L-GaL: L-galactono-1,4-lactone; L-GaA: L-galactonic acid; ++: more than 10 mM; + 10 mM or less; nd: not detectable

Claims

1. A process for the production of L-aldonolactone from L-aldohexose, especially for producing L-gulono-1,4-lactone or L-gulonic acid from L-gulose and producing L-galactono-1,4-lactone or L-galactonic acid from L-galactose by a microorganism capable of producing L-aldonolactone from L- aldohexose, and, optionally, isolating the L-aldonolactone from the reaction mixture.

2. The process of claim 1 wherein the microorganism is selected from the genera *Pseudomonas* or *Gluconobacter*, e.g. the microorganism is *P. putida*, e.g., *P. putida* ATCC 21812, or *G. oxydans*, e.g., *G. oxydans* IFO 3293.

3. The process of claim 1 wherein the microorganism is used in a growing culture or a resting cell reaction.

4. The process of claim 1, wherein the process is conducted at a pH in the range of from 1 to 9, at a temperature in the range of from 13°C to 45°C, for 1 to 120 hours.

5. The process of claim 1, wherein the process is conducted at a pH in the range of from 2 to 8, at a temperature in the range of from 18°C to 42°C, for 1 to 120 hours.

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